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Immunological Activities of Isoprinosine Inhibition on Viral Infections Inhuman

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Isoprinosineis a combination of inosine used as antiviral drug without effect on viral particle itself, but instead only and acts as on immunostimulant and also acts indirectly by activation of immune cells. Aim of this study was to determine level of interferon-alpha (INF-á) withparainfluenza viruses HPIV-2, and adenoviruses HAdV-2 replication. In the present study, cytotoxic effect of isoprinosine was assessed using A549 cell line exposed to different concentrations of compound (isoprinosine: 50-800ìg/mL) for 48 hours. Cytotoxic effect was examined visually using light, inverted microscopy Olympus CK2 under 400x magnification and by the MTT colorimetric assay. The yield reduction assay (YRA), which evaluates the ability of the isoprinosine(50-800 ìg/mL) to inhibit virus multiplication in cell cultures, was applied. The cytopathic effect of the virus was evaluated 48 h after infection of A549 cell cultures with viruses by means of light, inverted microscopy. The YRA method was used to determine the 50% end point (IC_{50}) in the presence of Isoprinosine with the controlled one. MTT cytotoxicity assay confirmed microscopic observations.There were no morphological changes, as assessed visually, in cell cultures treated with isoprinosine. After conducting the experiments and analyzing the results we noticed that higher concentrations of isoprinosinestrongly inhibited multiplication of all viruses. HPIV-2 and HAdV-2 showed the highest sensitivity to the antiviral activity of isoprinosine as compared with the control, however, increasing concentrations of isoprinosineup to 800 ìg /ml slightly enhanced the antiviral activity of 400 ìg/ml isoprinosine. Our study was conducted that HAdV-2 and HPIV-2 have the highest sensitivity to the antiviral activity of isoprinosine from all tested viral strains.

Keywords: Adenoviruses, antiviral activity, Isoprinosine, parainfluenza viruses.

Antiviraldrugs substances exhibiting interference with individual replication stages virusesarea need to search because of the limited numberofthem. The conventional "one virus - one drug" model actually makes the most of the possibilities therapeutic. Searching can therefore be one of the strategies for controlling viral infections takes into account pleiotropic effect and strengthens the antiviral reaction it also includes substances modulating the immune system[1].

Interferon (IFN) is a cytokine involved in the body's immune response at sites and at systemic level, and involved in reducing infection viral[2]. IFN was described in 1957 by Issacs and Lindemann as a factor that interferes with viral replication, often the most common protective one the impact of interference on herpes infection of the set as early as the 1930sas so-called Margassi phenomenon[3]. Produced by a cell of the immune system IFN-á includes pleiotropic antiviral activity. It binds to

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specific onesreceptors for cells and analysis based on antiviral proteins.Viral dsRNA is an inducer of IFN-á production. In infected cells antiviral effect of interferon generated with enzyme activity: 2', 5' -oligoadenylsynthetase and protein kinase R(PKR), resulting in inhibition of synthesis proteins by PKR phosphorylation and degradation of viral[4]. IFN-á stimulates also cellular response by activating effector cards. Stimulates for example, pre-NK cells for differentiation into NK cells and activates early, natural protection against infection. INF-á together with IFN-á increase expression MHC class I molecules, which improves the ability of the cell to present antigen. State antiviral, stimulated by the presence of IFN-á in the infected cell, lasts about 2-3 days[5]. Despite the fact that about 60 antiviral drugs are currently available, closehalf of them are registered for the treatment of HIV infection. Others are usedin the treatment of viral hepatitis (HBV, HCV), human infectionsherpesviruses and influenza viruses. There is still a lack of possibilities to control many important onesclinically and epidemiologically viral infections[6]. Infections caused by adenoviruses or parainfluenza viruses are common, usually mild, self-limiting infections. However, the immune system can cause serious complications.Adenoviruses initially infect or reactivate in 20-50% of people with immunosuppression,causing organ or generalized infections[7]. Parainfluenza viruses in situations of weakened immune mechanismsthe body cause acute pneumonia, requiring artificial ventilation and in partcases (5-35%) resulting in death[8]. None of the previously known preparationsantiviral has no registration for the treatment of infections with these viruses.Isoprinosineis a complex of inosine, p-acetamidobenzoate and diamidopropanol - a drug belonging to itto the group of general purpose antiviral pharmaceuticals[9]. Isoprinosineexceptantiviral activity has confirmed in both in vitro and in clinical trials, an immunomodulatory effect[10]. It is likely that this compound is involved in inhibitionviral RNA synthesis. Stimulates the immune system affecting puberty as wellactivation of lymphocytes. Numerous studies (in vitro and in vivo) have been shown to increase Isoprinosineproduction of cytokines such as interleukin (IL-1,IL-2 and IL-12),interferon á (IFN-á), TNF-á and interferon ã (IFN-ã), inhibits IL-10 production, enhances the effectcytotoxic (natural killer;NK cells) and stimulates chemotaxis and phagocytosis[11]. The aim of the study was to assess the effect of interferon-á and Isoprinosineon titers infectious RNA viruses: parainfluenza viruses(human parainfluenza 2 virus; HPIV-2) and human C adenoviruses(human adenovirus 2; HAdV-2)in vitro.

MATERIAL AND METHODS

RNA viruses pathogenic to humans were used in the study: adenovirus 2(HAdV-2) and parainfluenza virus type 2 (HPIV-2). Viruses propagated in A549 cell line (ATCC® CCL-185MT). Cells were cultured in medium Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific) supplemented with 10% calf fetalbovineserum (FBS; ThermoFisher Scientific)and a1% penicillin mixture /streptomycin and antimicotic solution (BI; Biological Industries) and 5% atmosphere . For reproduction viruses, and evaluation of antiviral activity, reduced fluid medium was used up to 2% FBS concentration. Interferon-á (Switzerland) was added to the culture at concentrations of 100 and 1000 IU/ml. Interferon doses were selected based on literature analysis and own experience. Isoprinosine(BI; Biological Industries) in a dose 5.0 mg were suspended in 5.0 mL of Phosphate-buffered saline (PBS,PH=6.9), filtered using a millipore filter (Filter Unit, 0.2 ìm). Prepared non-toxic for cell culture concentration: 50-800 ìg / ml. The selection of doses was determined on the basis of previously conducted and published research results, in which using the method qualitative using an inverted optical microscope (OLYMPUS),imageenlarged by 400x and by quantitative method using the tetrazole salt reduction testin cell mitochondria, MTT Cell Proliferation Assay (ATCC bioproducts ™, USA) has been shown to be non-toxic isoprinosineactivity on A549 culture , HEp-2 and HEL 299 cell lines [(12)].Antiviral activity testing involved infection of A549 cells (1x105 cells / ml) with each virus at a dose of 100 $TCID_{50}$ / ml (Tissue Culture Infectious Dose). After 60 min incubation of the virus with cells in micro plates (in a volume of 0.2 ml in flat-bottom 96-well plates, (MEDLAB-PRODUCTS) twice cultures were washed with PBS to remove non-cell-associated viruses. Then, infected cells were added:

1. D-MEM medium (virus control).
2. Isoprinosine at concentrations from 50 to 800 ìg / ml in DMEM liquid medium.
3. IFN-á at a concentration of 100 or 1000 IU / ml.
4. Isoprinosine and IFN-á in w/w concentrations.

The exposure time in each system was 48 hours the antiviral effect was determined by the method of reduction of infectious titers (YRA – yield reduction assay). The virus infectious titer is expressed in log10 $TCID_{50}$ / ml. Significance was determined differences between average virus titers,cell culture microplates were frozen and thawed three times(to release viruses from cells), centrifuged for 10 minutes (3000 rpm, temperature 4 °C).The supernatant was used to assess viral load according to the Reed-Muench method[13].

Statistical analysis

Student's t-test was used for related variables, the significance level $P < 0.05$ was adopted.The results were statistically evaluated using the Pearson correlation methodmeasuring the relationship between isoprinosinedoses and viral load and INF-á. Correlation coefficient valuein the range of 0.4-0.7 interpreted as a moderate relationship, 0.7-0.9 relationshipsignificant above 0.9 as a very strong relationship.

RESULTS

There was no toxicity to Isoprinosine at concentrations between 50 and 800ìg / ml for culturing.IFN-á has been shown to significantly inhibit the multiplication of these viruses in research in vitro ($P < 0.05$). The reduction of infectious titers was linearly dependent on concentration IFN-á. The concentration of 100 IU / ml, 1000IU / ml andisoprinosine in concentrations 200ìg / ml and 800ìg / ml. IFN-á reduced the infectious titreHPIV-2, andHAdV-2respectively by more strongly inhibited replication resulting in 100 IU / ml , 1000IU / ml and isoprinosine in concentrations 50ìg / ml,100ìg / ml and 400ìg / mlcompared with acontrol.Analyzing the Pearson correlation coefficient was demonstratedsignificant ($P < 0.05$) dependence of the infectious titreHPIV-2and HAdV-2on the dose of isoprinosinein culturesA549. Similarly, a high correlation coefficient (however not statistically significant) was calculatedfor other RNA viruses: HPIV-2and HAdV-2.isoprinosine most strongly reduced HAdV-2infectious titers compared to control , but increasedisoprinosine concentrations up to 800 ìg / ml slightly enhanced the antiviral effect of 50 ìg / ml isoprinosine.as shows table(1)In vitro experiments have shown that isoprinosine even more stronglyreduces infectious adenovirus titers in the presence of interferon-á (IFN-á). Simultaneousthe addition of 100 IU / mL and 1000 IU / mL IFN-á and isoprinosine to A549 infected cells with resultedreduction of IC_{50} values (media concentration of the inhibitor that inhibits infectious titers by 50%)from 9051ìg / mL to 8931ìg / mL forHPIV-2respectively,9873.75ìg /

Table 1. Reduction of infectious titers of parainfluenza viruses and adenoviruses examinedunder the influence of different doses of Isoprinosine in vitro culture of A549 cells

Parameter		Isoprinosine concentration				
		50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
IFN-α 100 IU/ml	HPIV-2	1.86E±0.6	5.00E±0.4	4.99E±0.5	3.35E±0.6	6.95E±0.5
	HAdV-2	1.08E±0.5	1.85E±0.4	8.01E±4.04	2.32 E±0.4	7.92E±0.3
IFN-α 1000 IU/ml	HPIV-2	1.03E±0.5	2.32E±0.4	5.05E±6	2.35E±0.5	4.06E±0.4
	HAdV-2	1.06E±0.5	2.o6E±0.05	7.92E±3.1	2.32E±0.4	7.56E±0.3

Table 2. In vitro antiviral activity of isoprinosinewith interferon alpha, during expressed as IC_{50}

Parameter	IFN-α 100 IU/ml	IFN-α 1000 IU/ml
IC_{50} (min-max)[ìg/ml] of HPIV-2	9051 (845-15517)	8931 (7320-13171)
IC_{50}(min-max)[ìg/ml] of HAdV-2	9873.75 (842,5-1271,8)	7816.5 (577,6-9201,9)

mL to7816.5ìg / mL and HAdV-2, respectively as show table (2).

DISCUSSION

Isoprinosinehas antiviral activity, it also has confirmed immunomodulatory effect[14]. Its effectiveness has been described in randomizedand double-blind clinical trials. Deroñ analyzing the resultsGinsberg *et al*[15]. experiments confirm good drug tolerance in the macroorganism. After reaching a high concentration in tissues, isoprinosine is metabolized to uric acidand completely eliminated by the kidneys[16]. The study showed thatthat Isoprinosine is not cytotoxic to A549 cells. isoprinosineat a dose of 800 ìg / ml, it also does not change the morphology and does not affect biological activity(in the MTT test) HEp-2 and HEL 299 cell lines[17]. Assessing the effect of isoprinosineon the replication of parainfluenza, andadenoviruses have been shown to isoprinosine after 48 hours.Adenoviruses (HAdV-2) have also been shown to be of the highest susceptibilityon the antiviral effect of Isoprinosine among all usedin the study of virus strains. In vitro experiments have shown that isoprinosine even more stronglyreduces infectious adenovirus titers in the presence of interferon-á (IFN-á). Simultaneousthe addition of 100 IU / mL and 100 IU / mL IFN-á and isoprinosine to A549 infected cells with resultedreduction of IC_{50} values (media concentration of the inhibitor that inhibits infectious titers by 50%)from 9051ìg / mL to 8931ìg / mL forHPIV-2respectively,9873.75ìg / mL to7816.5ìg / mL and HAdV-2, respectively. Enhancement of the action of Isoprinosine in the presence of INF-á has been demonstratedalso towards the reference strain (Human Herpesvirus1)HHV-1McIntyre strain[18]. It also turns out to be simultaneousadministration of IFN-á and isoprinosine results in improvement of the neurological condition of patients with subacutesclerosing encephalitis (a complication after mumps infection) and helpsconventional treatments for local human infectionspapillomavirus (HPV)[19]. Due to the limited ability to control many viral infections it appearsreasonable implementation of subsequent experiments to confirm the effectiveness of isoprinosinein inhibiting the replication of clinically or epidemiologically important virusespathogenic to humans.

Parainfluenza viruses are responsible for respiratory infections that occur with varying severity, depending on the type of virus and the immune function host. On the other hand, adenovirus cause infections in people with various pictures clinical, ranging from upper respiratory tract infections to myositis cardiac or central nervous system infections[20]. In this study, IFN-á and isoprinosine have been shown to be added jointly to the virus infected A549 culture RNA reduced adenovirus and parainfluenza virus 2 infectious. Both drugs have documented effects therapeutic also against other viruses[3, 5, 6, 10, 21]. isoprinosine has been shown at therapeutic doses has no toxic effect; good also has been confirmed drug tolerance[22]. Certainly, the number of publications on biological activity isoprinosine and pharmacokinetic properties of the drug is limited; studies done at various centers confirm that isoprinosine enhances the cellular immune response in cell culture in vivo. Affects the maturation and activation of lymphocytes, increases production of cytokines and stimulates phagocytosis and chemo taxis[23]. isoprinosine is also characterized by pleiotropic antiviral activity, which is, however, secondary to its immunomodulatory properties[24]. Linhares *et al*.2003[25] showed in vitro experiments that isoprinosine is a synthesis inhibitor Rotavirus RNA. The beneficial effects of isoprinosine in controlling were also highlighted acute viral encephalitis, skin and mucous membrane infections in infected persons herpes simplex viruses, as well as hepatitis A[26]. Ochocka *et al*.2006[27] describe good isoprinosine tolerance and reduction of illness time due to human alpha herpesvirus infection in children with acute lymphoblastic leukemia treated isoprinosine. Positive pharmacological effect of isoprinosine has also been demonstrated in the treatment of symptomatic human papillomavirus infection in women[28]. In a study conducted by Rhoades*et al.,*[29] isoprinosine was shown to influence the dynamics of HIV infection; reduces the level of reverse traxriptase and reduces expression p24 and gp120 on the surface of HIV infected lymphocytes. In addition, it was observed that viability, as well as the number of CD4 + cells and the ratio CD4 + / CD8 + are higher in culture

cell treated with isoprinosinecompared to HIV infected and non-exposed isoprinosine cells[4,30]. In vitro studies have been observed antiviral activity of isoprinosine and inhibiting the multiplication of viruses as a result of the combined administration of isoprinosine and interferon-á. Isoprinosine and IFN-á have been shown to reduce infectious titers of DNA viruses such as human adenoviruses and herpes viruses common[31]. In patients with sub-acute sclerosing encephalitis (SSPE), associated with measles virus infection, combination therapy is recommended isoprinosine with interferon, due to their likely synergistic actions[32]. Currently, there is no possibility of fully effective causal treatment of infections caused by viruses used in the RNA study. It is also not available specific prevention. Therefore, when confirmed in vivo, demonstrated in an antiviral study of co-administered interferon and isoprinosine, it is possible to include such a combination in controlling infections viral[33].

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